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BIOSYNTHESIS OF DULCITOL IN *EUONYMUS JAPONICA*

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Abstract—$^{14}CO_2$, glucose-1-^{14}C and galactose-1-^{14}C were administered to leaves of *E. japonica* and samples were taken for time periods ranging from 0·5 hr to 24 hr. For each time period the absolute activity of the glucose, galactose and dulcitol pools was determined. Such studies demonstrated that the primary product of photosynthesis, glucose, is epimerized to galactose which is then reduced to dulcitol.

INTRODUCTION

THE PRESENCE of dulcitol (galactitol) in many plant species has been well documented.[1–4] Although the distribution, physiology and biochemistry of some acyclic polyols has been studied extensively,[5–10] only recently has the study of the biochemistry of dulcitol begun.[11] This previous investigation showed that dulcitol was formed from galactose. In the present investigation, the complete pathway for the synthesis of dulcitol from photosynthesis was determined by the study of the distribution of label following the administration of the labelled substrates.

RESULTS

The activity of the glucose, galactose and dulcitol pools following the administration of $^{14}CO_2$, glucose-1-^{14}C and galactose-1-^{14}C is given in Tables 1–6. The activity of these pools are expressed as a percentage of the total activity (μCi/mM). Since the amount of labelled glucose and galactose introduced into the individual leaf samples varied, the specific activity of the carbohydrate pools is expressed on the basis of 100 000 dpm in the methanolic extract. Samples obtained in the $^{14}CO_2$ fixation study are identified by the length of time the leaf samples were exposed to $^{14}CO_2$ plus the length of the $^{12}CO_2$ flush period. The difference in the activity of corresponding samples in the two experiments is due to the difference in $^{14}CO_2$ concentration employed in the two experiments. Samples infiltrated with solutions of glucose-1-^{14}C and galactose-1-^{14}C are identified by the length of time the leaf samples were allowed to metabolise the exogenous substrates.

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DISCUSSION

$^{14}CO_2$ *Fixation*

The results in Table 1 indicate that the percentage of the label found in the glucose pool is greatest in the samples with the shortest flush periods. As the length of the flush period is increased, the amount of labelled glucose decreases. The galactose pool, however, reaches its peak activity only after a flush period of 2–4 hr. The percentage of the label in the dulcitol pool was greatest after a flush period of 24 hr in samples exposed to photosynthetic conditions. Although the proportion of the label found in the galactose and dulcitol pools is much smaller than the glucose pool, the results indicate that some of the glucose synthesized from the fixation of CO_2 is converted to galactose which is then reduced to dulcitol.

TABLE 1. PERCENTAGE OF TOTAL ACTIVITY RESIDING IN CARBOHYDRATE POOLS FOLLOWING $^{14}CO_2$ INCORPORATION

Hours in $^{14}CO_2$ $^{12}CO_2$	Total activity in tissue (dpm $\times 10^{-6}$)	% activity in Dulcitol	% activity in Galactose	% activity in Glucose
Run No. I				
1 + 1	51·65	0·95	1·98	26·3
1 + 2	52·33	1·07	1·45	13·3
1 + 4	57·10	2·48	1·69	13·0
1 + 8	59·26	1·75	1·19	11·1
1 + 24	37·20	2·56	0·84	5·8
Light				
2 + 24	59·99	3·64	1·44	10·9
Dark				
Stems	16·75	2·08	0·90	8·7
Run No. II				
1 + 1	24·15	1·10	1·40	12·4
1 + 2	23·23	1·93	1·55	12·6
1 + 4	20·15	2·16	1·29	11·7
1 + 8	31·61	1·50	0·79	7·2
1 + 24	14·93	2·83	0·63	4·5
Light				
1 + 24	20·61	4·24	1·41	9·8
Dark				
Stems	6·94	3·02	0·82	—

Leaf samples, uniform in size, contained approximately equal quantities of dulcitol. Since labelled dulcitol was synthesized following exposure to $^{14}CO_2$, some dulcitol must continuously leave the dulcitol pool (Tables 2 and 3). The absolute activity of the dulcitol pool decreases after a flush period of 4 hr in the first experiment as does the specific activity of the dulcitol pool. The decrease in absolute activity of the dulcitol pool after a flush period of 8 hr in the second experiment may have been caused by a decrease in size of the leaf sample, since the specific activity of the dulcitol pool in this sample continued to increase.

TABLE 2. DISTRIBUTION OF ACTIVITY IN CARBOHYDRATE POOLS FOLLOWING $^{14}CO_2$ INCORPORATION

Hours in $^{14}CO_2$ $^{12}CO_2$	Activity (dpm × 10^{-3}) Galactose	Dulcitol	Glucose
Run No. I			
1 + 1	1246	494	10501
1 + 2	763	560	6985
1 + 4	965	1420	7479
1 + 8	710	1041	6620
1 + 24	314	954	2171
Light			
1 + 24	866	2186	6552
Dark			
Stems	150	348	1470
Run No. II			
1 + 1	338	266	3008
1 + 2	360	450	2936
1 + 4	260	435	2375
1 + 8	249	475	2287
1 + 24	94	423	675
Light			
1 + 24	292	875	2032
Dark			
Stems	57	210	

TABLE 3. SPECIFIC ACTIVITY OF CARBOHYDRATE POOLS FOLLOWING $^{14}CO_2$ INCORPORATION

Hours in $^{14}CO_2$ $^{12}CO_2$	Specific activity (μC/mM) Galactose	Dulcitol	Glucose
Run No. I			
1 + 1	110·7	1·63	20·7
1 + 2	58·2	1·90	131·0
1 + 4	89·0	5·33	138·4
1 + 8	47·7	3·86	99·3
1 + 24	24·4	3·33	49·7
Light			
1 + 24	52·3	7·24	104·6
Dark			
Stems	2·4	0·61	21·9
Run No. II			
1 + 1	25·7	1·02	40·8
1 + 2	53·2	1·33	64·5
1 + 4	22·4	1·35	28·8
1 + 8	10·6	1·80	27·8
1 + 24	9·4	1·86	13·7
Light			
1 + 24	42·8	3·62	49·1
Dark			
Stems	—	0·49	—

Leaf samples which were placed in the dark for 24 hr were removed from the stems at the same time as the samples with a flush period of 1 hr. A comparison of the percentage activity and the specific activity of the dulcitol pools in these samples, however, indicates a much higher dulcitol activity in the samples which were placed under respiratory conditions. The synthesis of dulcitol must therefore proceed under conditions favoring respiration. A comparison of the activity of the dulcitol pools of samples flushed for periods of 24 hr under respiratory conditions to those flushed for a similar period under conditions of photosynthesis reveals a much smaller activity in the latter. This phenomena may be the result of, (1) an increased rate of synthesis of dulcitol under conditions of respiration, or (2) a decrease in the rate of utilization of dulcitol under respiratory conditions.

Table 3 shows that the specific activity of glucose and galactose reach peak values at the same time. The specific activity of dulcitol in the first experiment reaches its peak value at the same time as, in the second experiment, the specific activity of dulcitol rises continuously with time. This indicates that while the epimerization of glucose to galactose proceeds smoothly, the reduction of galactose to dulcitol may be determined by the rate of utilization of dulcitol.

Glucose-1-^{14}C Utilization

Generally, the results obtained following glucose-1-^{14}C infiltration (Table 4) are similar to those obtained from the fixation of $^{14}CO_2$. The specific activity of glucose behaves somewhat erratically, although this is to be expected since the utilization of glucose will be

TABLE 4. SPECIFIC ACTIVITY OF CARBOHYDRATE POOLS FOLLOWING GLUCOSE-1-^{14}C INFILTRATION

Time (hr)	Specific activity of carbohydrates (μC/mM)* Galactose	Dulcitol	Glucose
Run No. I			
0·5	0·31	0·020	0·43
1	1·03	0·098	0·39
2	1·50	0·150	1·27
4	1·13	0·139	0·56
8	1·67	0·076	0·31
24 Light	2·52	0·081	0·33
24 Dark	—	—	0·44
Run No. II			
0·5	0·65	0·038	0·23
1	0·42	0·139	0·66
2	0·49	0·055	0·49
4	5·34	0·444	1·07
8	1·31	0·063	0·48
24 Light	0·61	0·045	4·56
24 Dark	—	—	3·10

* Based on standardized 100 000 dpm in methanolic extract.

largely dependant on the requirement for glucose in the individual leaf samples. The behavior of the specific activity of the galactose pool, however, is almost identical to specific activity following $^{14}CO_2$ fixation with the peak values being reached 2–4 hr following infiltration. Specific activity of the dulcitol pool follows the pattern of $^{14}CO_2$ fixation (experiment No. 1). This indicates that approximately 4 hr after the infiltration process, the utilization of dulcitol was greater than the rate of synthesis of this polyol.

Leaf samples which were infiltrated with glucose-1-^{14}C and then placed immediately in the dark contain galactose and dulcitol pools with no detectable activity. This indicates that glucose was not epimerized to galactose under respiratory conditions. The increased activity of the dulcitol pool in leaves which had been exposed to $^{14}CO_2$ and then placed in the dark may therefore be the result of decreased utilization of the dulcitol pool.

Galactose-1-^{14}C *Utilization*

The results in Tables 5 and 6 indicate that although some of the galactose is converted to glucose the majority is reduced to dulcitol. As expected the amount of activity in the galactose pool decreased steadily throughout the course of the experiment. Expression of the

TABLE 5. PERCENTAGE C^{14} ACTIVITY IN THE CARBOHYDRATE POOLS FOLLOWING GALACTOSE-1-^{14}C INFILTRATION

Time (hr)	% of ^{14}C activity Dulcitol	Galactose	Glucose	Total activity in tissue (dpm × $^{-}10^{3}$)*
Run No. I				
0·5	17·5	21·7	7·7	234
1	29·9	19·8	7·2	444
2	27·5	10·3	2·8	511
4	29·4	6·5	6·5	247
8	58·1	5·7	7·6	260
24	19·6	3·0	3·4	370
Light				
24	25·3	8·1	6·6	194
Dark				
Run No. II				
0·5	29·9	36·6	13·3	123
1	26·5	17·8	6·9	195
2	35·2	13·0	7·4	153
4	41·1	22·7	21·3	125
8	43·3	10·0	10·4	254
24	24·6	2·8	4·4	322
Light				
24	31·6	4·3	5·3	307
Dark				

* Tissue total = dpm of methanolic extract + dpm residue.

activity of the dulcitol pool as a percentage of the total activity or as specific activity again illustrates the state of flux of the dulcitol pool. While the specific activity of the dulcitol pool reaches its peak value 4 hr after infiltration, the percentage activity residing in this pool reaches its peak value 8 hr after infiltration. This indicates that the rate of utilization of labelled dulcitol exceeds the rate of dulcitol synthesis after 4 hr.

TABLE 6. SPECIFIC ACTIVITY OF CARBOHYDRATE POOLS FOLLOWING GALACTOSE-1-^{14}C INFILTRATION

Time (hr)	Specific activity of carbohydrates (μC/mM)* Galactose	Dulcitol	Glucose
Run No. I			
0·5	1·32	0·059	0·132
1	1·53	0·092	1·288
2	1·19	0·091	0·100
4	0·88	0·232	0·267
8	0·52	0·200	0·380
24 Light	0·13	0·084	0·037
24 Dark	0·51	0·126	0·163
Run No. II			
0·5	2·09	0·106	0·334
1	1·15	0·066	0·128
2	1·49	0·151	0·318
4	4·64	0·395	1·494
8	0·90	0·171	0·420
24 Light	0·16	0·123	0·053
24 Dark	0·23	0·145	0·091

* Based on standardized 100 000 dpm in methanolic extract.

Comparison of the specific activity or per cent activity of the galactose pools and dulcitol pools of samples exposed to photosynthetic or respiratory conditions yields activities higher in both pools under respiratory conditions. The presence of labelled dulcitol in samples placed in the dark indicates that the reduction of galactose to dulcitol is independent of conditions of illumination.

The results from the incorporation of all three substrates therefore indicates that glucose is not converted to galactose under conditions of respiration but that the galactose present in the galactose pool under these conditions can be reduced to dulcitol. The consistently higher activity of the dulcitol pool of samples placed under respiratory conditions therefore indicates a decrease in the rate of metabolism of dulcitol.

EXPERIMENTAL

Cultivation of plants. The plants were grown in 25 cm flower pots containing sandy loam in an environment control room under the following conditions: light—16 hr at a temp. of 26 ± 1° and a relative humidity of 65 ± 5%; dark—8 hr at a temp. of 10 ± 1° and a relative humidity of 75 ± 5%.

Procedure for photosynthetic experiments. For each experiment, 6 spurs, each bearing 6 leaves were cut from the plant. The apparatus described by Andrews and Hough was used.[12] The first experiment employed 315·3 mg $Ba^{12}CO_3$ plus 33·8 mg $Ba^{14}CO_3$ while 332·1 mg $Ba^{12}CO_3$ plus 17·0 mg was used in the second. Steady illumination was provided in an apparatus 61 cm square. On both sides of this apparatus were mounted 7, 46-cm Cool White and 9, 46-cm Gro-Lux fluorescent light tubes. Following 1 hr exposure to $^{14}CO_2$, the atmosphere inside the desiccator was drawn through a $Ba(OH)_2$ solution while air was simultaneously allowed to enter the desiccator. This process was allowed to proceed for 1 hr after which the leaf samples were removed from the desiccator and placed on the floor of the illumination apparatus.

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Procedure for infiltration experiments. 42 leaves (6 leaves for each time period) were used. Glucose-1-^{14}C and galactose-1-^{14}C (50 μCi, 3·0 mCi/mM, Amersham/Searle) were incorporated into the leaves by dissolving each in 150 ml H_2O and vacuum infiltrating the leaves in a 5 l. desiccator at 10 mm Hg. After infiltration the leaves were bathed in distilled water and placed in the illumination apparatus described above.

Extraction. Leaves were placed in 25 × 80 mm cellulose extraction thimbles, frozen with liquid N_2 and ground with an aluminum rod. The thimbles were then placed in a Soxhlet and extracted with methanol. After extraction, the methanol was evaporated and the carbohydrates were dissolved in pyridine. Silylation was carried out using hexamethyldisilazane and trichlorosilane.[13]

Separation and collection. The glucose, galactose and dulcitol peaks were identified by an enrichment procedure in which individual silylated carbohydrates were added to the silylated solution, and the solution re-examined by GLC. Peak identity was confirmed by the addition of a labelled carbohydrate to the extract solution and the collection of this labelled sugar by the collection of the peak(s) previously designated. A quantitative estimate of the amount of sugars present in the extract was obtained using an internal arabinitol standard and determining the area of the peaks by triangulation.

A Microtek Model 220 with a Packard Model 852 fraction collector was used for the preparative GLC. The following conditions were employed: column—length, 162·5 cm, i.d., 6 mm; liquid phase—SE 52, 3% wt; support—Chromosorb W, DMCS treated, acid washed, 70/80 mesh size; detector—Flame ionization; pressure—40 psi; flow rates; Hydrogen, 40 cm^3/min, Air, 28 l./hr. temp.: column—120–240° programmed at 2°/min, detector, 245°; inlet, 220°; splitting arm—250°; carrier gas—N_2, flow rates: scavange: 21 cm^3/min; split: 71 cm^3/min. The carbohydrates were collected on *p*-terphenyl collection vials as they eluted.

Determination of activity. All samples were counted in a Nuclear-Chicago Model Mark 1 liquid scintillation counter. 15 ml fluor solution (5 ml dioxane containing 1·2% PPO and 0·02% dimethyl POPOP plus 10 ml toluene containing 0·6% PPO and 0·01% demethyl POPOP)[14] was used to count a 0·5-ml aliquot of the methanolic extract. The sugars collected on *p*-terphenyl were counted without flour but the count rate was multiplied by a factor, previously determined, to compensate for the difference in count rates obtained using the two methods.

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